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Occurrence and antibiotic resistance of *Campylobacter jejuni* and *Campylobacter coli* in retail broiler chicken

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Abstract: Broiler part samples (80 fresh and 80 chilled) were examined for the prevalence and numbers of *C. jejuni* and *C. coli* by employing most-probable-number (MPN) and polymerase chain reaction (PCR) techniques. The prevalence of the bacteria was high where *C. jejuni* was detected in 92.5% fresh and 53.8% chilled samples while *C. coli* in 80.0% fresh and 56.3% chilled. The number of these bacteria in the positive fresh and chilled samples was from 3 to more than 2400 MPN/g and from 3 to 290 MPN/g, respectively. Antibiotic resistance test (using Kirby-Bauer disc diffusion method) on 10 *C. jejuni* and 13 *C. coli* isolates toward ampicillin, tobramycin, enrofloxacin, ciprofloxacin, tetracycline, cephalothin, gentamicin and norfloxacin revealed high resistance toward all antibiotics (20.0% - 100.0%). All isolates were resistant to at least two antibiotics. This study highlights the potential of multidrug-resistant *C. jejuni* and *C. coli* transmission to humans through fresh and chilled broiler parts. Consecutive studies with bigger sample sizes and covering all over Malaysia are warranted in future.

Keywords: Broiler chicken, *C. jejuni*, *C. coli*, prevalence, antibiotic resistance

Introduction

Poultry meat is often suggested as the major source of campylobacteriosis (Andersen *et al*., 2006; Peyrat *et al*., 2008). There are many species of *Campylobacter*, but only the thermotolerant *C. jejuni* and *C. coli* are by far the most common in infecting humans (Yan *et al*., 2005). A low dose of *Campylobacter* (500-800 organisms) is sufficient to cause infections (Konkel *et al*., 2001).

Campylobacter has not only emerged as a hazard to consumers in relation to food poisoning, but also through the spread of antibiotic resistance (Cardinale *et al*., 2006). There has been a link between antibiotic use in food producing animals, emergence of antibiotic resistance in *E. coli, Salmonella*, Enterococci and *Campylobacter* in treated animals, and transfer of these resistant organisms (or their resistance genes) to humans through the food chain (Akinbowale *et al*., 2006). Resistance toward the antibiotics recommended for the treatment of *Campylobacter* infections increases the need for alternate antibiotics, which are limited (Hong *et al*., 2007).

Therefore, apart from the necessity to know the bacterial prevalence and numbers in retail chicken and implementing proper control measures in reducing them, it is also crucial to actively monitor the antibiotic resistance in order to obtain surveillance data and develop measures to extend the life of antibiotics used, which in return will protect the public health. Apart from the very few studies on *Campylobacter* done in Malaysia (Radu *et al*., 1996; Saleha, 2002; Tan *et al*., 2008; Tan *et al*., 2009; Tang *et al*., 2009), the current work can be included as one of the preliminary assessment of the *Campylobacter* spp. occurrence and antibiotic resistance. The objective of the study was to determine the prevalence, numbers and antibiotic resistance patterns of *C. jejuni* and *C. coli* isolates from broiler part samples.

Materials and Methods

Sample collection and preparation

Eighty fresh and 80 chilled broiler part samples were purchased randomly from a range of retail outlets (wet markets and supermarkets) in Serdang and Seri Kembangan (two adjacent towns in the state of Selangor). Samplings were done weekly for

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a period of eight months (January 2006 to August 2006). Eight types of parts were studied (thighs + drumsticks, breasts, livers, gizzards, feet, wings, keels and bishop's nose) for fresh and chilled samples, respectively, with ten samples for each type. Wet markets sell live broilers that are slaughtered, plucked and portioned upon purchase. Supermarkets sell broiler parts at 4°C in Styrofoam trays which are over-wrapped with polyvinylidene film. Samples were transported to the laboratory in separate insulated cool boxes containing ice packs and were processed within 2 hours of being collected.

Sample preparation was carried out by adapting the method of Whyte *et al.* (2004). Portions of each broiler part sample (skin and muscle) were collected to make 10 g, and were homogenized for 1 minute in a sterile stomacher bag containing 90 ml of the Bolton selective enrichment broth (Merck, Darmstadt, Germany).

Most-probable-number (MPN)

A ten-fold MPN dilution series (three dilutions with three tubes per dilution) was prepared from the homogenate in Bolton selective enrichment broth (Lindquist, 2001). A control tube consisting of Bolton selective enrichment broth was also prepared. The MPN tubes and control tube were incubated in anaerobic gas jars at 42°C for 48 h under microaerophilic conditions (5-7% O_2 and 8-10% CO_2) which was achieved using a gas generating sachet, Anaerocult C (Merck, Darmstadt, Germany). After incubation, the MPN tubes were observed for the presence or absence of growth. Genomic DNA from the enrichment in MPN tubes with the presence of growth was extracted using the boiling method (Mohran *et al*., 1998) and kept at 4°C to be used for species-specific PCR reaction.

Primers

Oligonucleotide primers, derived from the *hip* and *ceuE* gene, were used to detect *C. jejuni* and *C. coli*, respectively. The primers were synthesized by First Base Laboratories, Selangor, Malaysia. The sequences of the primers are shown in Table 1.

Polymerase chain reaction (PCR)

By adapting the method of Gonzalez et al. (1997), the optimized PCR assay was performed in a 25 µl volume containing 2.5 µl of 10x PCR buffer (50 mM KCl, 10 M Tris-HCl, pH 9.1), 0.5 µl of 10 mM dNTP, 1.25 µl of 50 mM $MgCl_2$, 0.25 µl of 0.625 units of *Taq* DNA polymerase (Vivantis Technologies, Selangor, Malaysia), 1.0 µl of each 10 µM primer, 16.5 µl of sterile distilled water and 2.0 µl of DNA template. Amplification reactions were performed in a Gene Amp PCR System 2400 thermocycler (Perkin Elmer) according to the optimized PCR amplification parameters shown in Table 2. Reference strains (positive controls) were obtained from the World

Table 1. Primer sets for *hip* and *ceuE* gene for detecting *C. jejuni* and *C. coli*, respectively

Gene	Oligonucleotide primer sequences	Reference	Target gene (amplicon size [bp])
hip	Forward: 5'-GAAGAGGGTTTGGGTGGTG-3'	Linton *et al*., 1997	735
	Reverse: 5'-GAAGAGGGTTTGGGTGGTG-3'		
ceuE	Forward: 5'-ATGAAAAAATATTTAGTTTTTGCA-3'	Gonzalez *et al*., 1997	894
	Reverse: 5'-ATTTTATTATTTGTAGCAGCG-3'		

Table 2. PCR amplification parameters

Step	Number of cycles	Temperature, °C (Duration of time, minute)	
		hip gene	*ceuE* gene
Initial denaturation	1	94(5)	94(2)
Denaturation		94(0.5)	94(0.5)
Primer annealing	30	58(0.5)	55(0.5)
Chain extension		72(0.5)	72(0.5)
Final extension	1	72(7)	72(5)

Health Organization (WHO). Meanwhile, PCR reaction mix without DNA template, amplified under the same condition as PCR reaction mix with DNA template, was used as negative controls.

PCR products were subjected to electrophoresis in 1.0% agarose gel at 90 V for 1 h in 1x Tris-Borate-EDTA (TBE) buffer. The agarose gels were stained in ethidium bromide (0.5 µg ml^{-1}) for 5 minutes and were viewed under an ultraviolet (UV) transilluminator (SynGene Gel Documentation System).

MPN-PCR analysis

A dilution tube was scored positive for *C. jejuni* and *C. coli* when a PCR product was obtained. The number of positive tubes from each of the dilution were counted and were expressed as a series of numbers separated by dashes, starting with the lowest dilution (Entis, 2002). A three-tube MPN table (Lindquist, 2001) was used to convert the series of numbers to MPN of *C. jejuni* or *C. coli* per gram, by multiplying the MPN in the middle set of tubes with the dilution factor of middle dilution (10^{-2}).

Isolation of C. jejuni and C. coli

A loopful of the *C. jejuni* and *C. coli* PCR-positive-enrichment was spread plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Merck, Darmstadt, Germany). The plates were incubated at 42°C for 48 h under microaerophilic conditions. Typical grey to light tan, flat to slightly raised and round with a diameter of 1-2 mm *Campylobacter*-like colonies (Donnison, 2003) were subcultured on mCCDA and confirmed to the species level (*C. jejuni* and *C. coli*) by PCR and kept as glycerol stocks at -20°C.

Antibiotic resistance test

Cultures of *C. jejuni* and *C. coli*, grown overnight in Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) at 42°C under microaerophilic conditions, were swabbed onto MH agar plates (Oxoid, Basingstoke, UK) using sterile cotton swabs. After swabbing, each inoculum was dried for 5 to 10 minutes. Standard antimicrobial susceptibility test discs (ampicillin [AMP, 10 µg/disc], tobramycin [TOB, 10 µg/disc], enrofloxacin [ENR, 5 µg/disc], ciprofloxacin [CIP, 5 µg/disc], tetracycline [TE, 30 µg/disc], cephalothin [KF, 30 µg/disc], gentamicin [CN, 10 µg/disc] and norfloxacin [NOR, 10 µg/disc] - Oxoid, Basingstoke, UK) were placed onto the surface of the plate using a disc dispenser. The plate was inverted and incubated at 37°C for 24 h under microaerophilic conditions. The diameter of the growth inhibition zones was measured and interpreted as resistant or susceptible to the respective antibiotic in accord with the zone diameter breakpoints of National Committee for Clinical Laboratory Standards (NCCLS) (1990; 1997-a; 1997-b; 2002).

Data analysis

All statistical analysis was performed using SAS statistical program (version 8.02; SAS Institute, Cary, North Carolina). The Z-test on two proportions was used to analyze the statistical difference ($P = 0.05$) in the *C. jejuni* and *C. coli* contamination rate, respectively: (i) between the total fresh and chilled broiler samples and (ii) among the total fresh and among the chilled broiler samples. Analysis using randomized complete block design (RCBD) was employed to test if (i) types of broiler parts affect the prevalence rate of *Campylobacter* spp. in fresh and chilled broiler samples and (ii) types of antibiotics influence resistance on *Campylobacter* spp.

Results

Occurrence of *C. jejuni* and *C. coli* were identified in all types of broiler part samples except chilled feet where no *C. jejuni* was detected (Table 3). A comparatively low prevalence of *C. coli* was found in chilled gizzards and feet (20% and 10%, respectively). *C. jejuni* was found in 60% of the chilled gizzards (Table 3). The rest of the fresh and chilled samples had 50% to 100% prevalence for both *C. jejuni* and *C. coli*. Both species were found significantly more prevalent in total fresh samples than in total chilled ($P < 0.05$) (Table 3). Incidence of *C. jejuni* was significantly higher than *C. coli* in the total fresh samples ($P < 0.05$); *C. coli* were found slightly more than *C. jejuni* in the total chilled samples but the difference was not significant ($P > 0.05$). Contamination with both species from 3 to more than 2400 MPN/g and from 3 to 290 MPN/g were detected in fresh and chilled samples, respectively (Table 3).

When both prevalence and MPN were taken into account, fresh keels were found to be contaminated the most with *C. jejuni* (100%, exceeding 2400 MPN/g) while fresh gizzards were contaminated the most with *C. coli* (80%, exceeding 2400 MPN/g). Chilled feet were least contaminated with these two species (*C. jejuni* [0%], *C. coli* [10%, 3-75 MPN/g]). Nevertheless, the analyses of RCBD showed that the types of broiler parts did not influence the prevalence rate of *Campylobacter* spp. in fresh and chilled broiler.

The resistance rate of the *Campylobacter* isolates against the eight types of antibiotics was generally high, from 20% to 100% (Table 4). Analysis of

Table 3. Prevalence and MPN of *C. jejuni* and *C. coli* in broiler parts

Chicken samples	Number of broiler samples	Number[1] (%)	
		Number[1] containing 3-75 MPN/g (%), 150-290 _MPN/g (%), > 2400 MPN/g (%)	
		C. jejuni	*C. coli*
Thighs + drumsticks	Fresh (n=10)	9 (90.0)	9 (90.0)
		9 (100.0), 0 (0), 0 (0)	9 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	6 (60.0)	8 (80.0)
		5 (83.3), 1 (16.7), 0 (0)	8 (100.0), 0 (0), 0 (0)
Breasts	Fresh (n=10)	10 (100.0)	10 (100.0)
		10 (100.0), 0 (0), 0 (0)	10 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	6 (60.0)	10 (100.0)
		6 (100.0), 0 (0), 0 (0)	10 (100.0), 0 (0), 0 (0)
Livers	Fresh (n=10)	9 (90.0)	6 (60.0)
		9 (100.0), 0 (0), 0 (0)	6 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	7 (70.0)	5 (50.0)
		7 (100.0), 0 (0), 0 (0)	5 (100.0), 0 (0), 0 (0)
Gizzards	Fresh (n=10)	9 (90.0)	8 (80.0)
		9 (100.0), 0 (0), 0 (0)	6 (75.0), 0 (0), 2 (25.0)
	Chilled (n=10)	6 (60.0)	2 (20.0)
		6 (100.0), 0 (0), 0 (0)	2 (100.0), 0 (0), 0 (0)
Feet	Fresh (n=10)	8 (80.0)	8 (80.0)
		6 (75.0), 2 (25.0), 0 (0)	7 (87.5), 1 (12.5), 0 (0)
	Chilled (n=10)	0 (0)	1 (10.0)
		-	1 (100.0), 0 (0), 0 (0)
Wings	Fresh (n=10)	10 (100.0)	7 (70.0)
		10 (100.0), 0 (0), 0 (0)	7 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	6 (60.0)	6 (60.0)
		5 (83.3), 1 (16.7), 0 (0)	6 (100.0), 0 (0), 0 (0)
Keels	Fresh (n=10)	10 (100.0)	9 (90.0)
		9 (90.0), 0 (0), 1 (10.0)	9 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	6 (60.0)	8 (80.0)
		6 (100.0), 0 (0), 0 (0)	8 (100.0), 0 (0), 0 (0)
Bishop's nose	Fresh (n=10)	9 (90.0)	7 (70.0)
		8 (88.9), 1 (11.1), 0 (0)	7 (100.0), 0 (0), 0 (0)
	Chilled (n=10)	6 (60.0)	5 (50.0)
		6 (100.0), 0 (0), 0 (0)	4 (80.0), 1 (20.0), 0 (0)
Total	Fresh (n=80)	74 (92.5[2])	64 (80.0[3])
		70 (94.6), 3 (4.1), 1 (1.4)	61 (95.3), 1 (1.6), 2 (3.1)
	Chilled (n=80)	43 (53.8[4])	45 (56.3[4])
		41 (95.3), 2 (4.7), 0 (0)	44 (97.8), 1 (2.2), 0 (0)

RCBD showed that the types of antibiotics influenced resistance on *Campylobacter* spp. In both species, resistance to β-lactams predominated while resistance to aminoglycosides was the lowest. Resistance to cephalothin was a common characteristic of all *Campylobacter* isolates examined (Table 5). Meanwhile, resistance to ampicillin was a common feature in *C. jejuni*, as was enrofloxacin to *C. coli*. Resistant pattern that can be connected to particular species was not found.

Discussion

A large number of *Campylobacter* spp. are harbored by the intestinal tract of chicken, especially the ceca and colon. During processing activities, where the tract may leak or rupture, its contents would be transferred to the skin of carcasses (Scherer *et al*., 2006-b). Fecal contamination of carcasses is hard to avoid during processing activities (Hong *et al*., 2007), but poor processing techniques and sanitation conditions would certainly increase it. Processing activities at the wet markets studied in this study were done at the same place repeatedly for every batch of flocks. The action of slaughtering, defeathering, eviscerating, washing, portioning and retailing at the same place would greatly enhance the contamination of broiler samples with *Campylobacter,* as evidenced in our previous study (Chai *et al*., 2008; Usha *et al*., 2010). When it comes to washing and portioning, all the carcasses were washed a few times inside the same few basins of unchanged water while unwashed cutting boards and knives were used repeatedly. The washing system practiced cannot successfully remove *Campylobacter* from carcasses. The suitable microenvironment of the skin enables the persistence and survival of *Campylobacter* in feather follicles at room temperature, 4°C or frozen condition (Lillard, 1988; Scherer *et al*., 2006-b).

The real situation in supermarkets is not revealed to the public, but contamination might have occurred at the processing or packaging unit. Dirty defeathered and degutted broiler carcasses are bathed together in a chill tank; increased organic load are usually detectable in the tanks depending on the overall efficiency of the carcass washing system, including number of washers and types (Consumers' Association of Penang [CAP], 2002; Keener *et al*., 2004). Although a certain amount of chlorine is added in the washing and chilling system, organic matter can rapidly inactivate them and if the chlorine were to be active, the skin may protect *Campylobacter* (Sánchez *et al*., 2002).

The prevalence of *C. jejuni* and *C. coli* in total chilled samples, respectively, were high but were significantly lesser than those in total fresh ($P < 0.05$) (Table 3). The viability of campylobacters is affected when they undergo stress including rapid changes in temperature, osmolarity, oxygen tension and nutrient deprivation; however, it is most likely that a microaerophilic atmosphere suitable for *Campylobacter*'s survival is created when chicken samples are packed in polyvinylidene film-over-wrapped Styrofoam trays and stored under chilled temperature (Wallace, 2003; Diergaardt *et al*., 2004; Sallam, 2007).

This study found more than 90% of PCR-positive fresh and chilled samples containing *C. jejuni* and *C. coli* from 3 to 75 MPN/g (Table 3). No MPN limit for *Campylobacter* (maximum number of *Campylobacter* allowed to be present on per gram of raw retail chicken) has been established in Malaysia so far. MPN, as most food safety microbiological criteria, suggests the possibility of hazard, not the hazard itself. However, reducing the possibility of hazard would certainly diminish the hazard.

Different studies identified different types of chicken parts as the most or least contaminated with *Campylobacter* (Kotula and Pandya, 1995; Sallam, 2007; Stoyanchev *et al*., 2007). The question of which type of chicken part is contaminated the most (regarding both prevalence and numbers) may depend on the extent of contamination that occurs during processing or packaging activities, resulting in irregular occurrence of *Campylobacter* spp. throughout the year. In this study, chilled feet at purchase had their outer layer peeled off leaving the samples too dry under the low temperature (4°C) storage, which might account for their very low and zero prevalence of *C. coli* and *C. jejuni*, respectively (Table 3).

The high rate of resistance in this study may reflect the unrestricted and indiscriminate use of antibiotics at subtherapeutic doses in broilers for prophylaxis, growth promotion, and infection treatment (Saleha, 2002; Olah *et al.*, 2006). Withdrawal period to allow antibiotics used to leave the broilers before they are slaughtered may not be practiced. Stressing agents such as antibiotics used in processing activities, for instance chilling water tank, may result in the selection of microflora too (Sánchez *et al*., 2002).

Generally, *Campylobacter* spp. are inherently resistant to β-lactams due to their ability to produce β-lactamases, low affinity binding of β-lactams to the target (penicillin-binding proteins [PBPs]), or failure of the drugs to penetrate the outer membrane porins (Engberg *et al*., 2006; Li *et al*., 2007). Cross-resistance among *Campylobacter* strains to enrofloxacin,

Table 4. Antibiotic resistance of *C. jejuni* and *C. coli*

Isolates / Antibiotics	*C. jejuni* (N = 10)		*C. coli* (N = 13)	
	Number[5]	%[5]	Number[5]	%[5]
Ampicillin	10	100.0	12	92.3
Cephalothin	10	100.0	13	100.0
Enrofloxacin	7	70.0	13	100.0
Ciprofloxacin	5	50.0	11	84.6
Norfloxacin	6	60.0	6	46.2
Tetracycline	8	80.0	10	76.9
Tobramycin	3	30.0	6	46.2
Gentamicin	2	20.0	5	38.5
Resistance to				
2 antibiotics	1	10.0	0	0.0
3 antibiotics	1	10.0	0	0.0
4 antibiotics	1	10.0	0	0.0
5 antibiotics	2	20.0	7	53.8
6 antibiotics	4	40.0	3	23.1
7 antibiotics	0	0.0	1	7.7
8 antibiotics	1	10.0	2	15.4

[5]Resistance isolates

Table 5. Antibiotic resistance patterns of *C. jejuni* and *C. coli* isolates

Resistance pattern (pattern number)	*C. jejuni*		*C. coli*	
	Number[6]	%[6]	Number[6]	%[6]
Amp Kf (1)	1	10.0	0	0.0
Amp Kf Te (2)	1	10.0	0	0.0
Amp Kf Te Cn (3)	1	10.0	0	0.0
Amp Kf Te Enr Tob (4)	1	10.0	0	0.0
Amp Kf Enr Cip Nor (5)	1	10.0	1	7.7
Amp Kf Te Enr Cip (6)	0	0.0	4	30.8
Amp Kf Enr Cip Tob (7)	0	0.0	1	7.7
Amp Kf Enr Cip Cn (8)	0	0.0	1	7.7
Amp Kf Te Enr Cip Nor (9)	4	40.0	1	7.7
Amp Kf Te Enr Nor Tob (10)	0	0.0	1	7.7
Amp Kf Te Enr Tob Cn (11)	0	0.0	1	7.7
Kf Te Enr Cip Nor Tob Cn (12)	0	0.0	1	7.7
Amp Kf Te Enr Cip Nor Tob Cn (13)	1	10.0	2	15.4

[6]Isolates having resistance pattern

ciprofloxacin, and other fluoroquinolones might also explain for the increasing number of resistance toward fluoroquinolones (Jacobs-Reitsma *et al*., 1994; Hong *et al*., 2007).

There is evidence indicating that *Campylobacter* strains are able to maintain tetracycline resistance over a longer period of time, even when the antibiotic is no longer used (Schuppers *et al.*, 2005). This could be one of the reasons for the high tetracycline resistance. Low level of resistance to aminoglycosides generally can be attributed to the rare usage of this group in the poultry industry either prophylactically or therapeutically due to its intramuscular route of administration, which may be impracticable for large-scale application (Rodrigo *et al*., 2007).

A lot of publications have associated multidrug resistance in campylobacters with the expression of the *cmeABC* efflux system (Lin *et al*., 2002; Fallon *et al*., 2003; Minihan *et al*., 2006; Olah *et al*., 2006). The efflux pump extrudes a broad range of structurally unrelated antibiotic agents (including fluoroquinolones, β-lactams, tetracycline, gentamicin) out of bacterial cells, and thus contributes to the intrinsic antibiotic resistance and high levels of multiple resistance (Lin *et al*., 2002; Poole, 2004; Olah *et al*., 2006).

So far no differences have been reported in the antibiotic resistance profiles of veterinary *Campylobacter* spp. (even from those isolated from food animal processing environments having substantially different processing regimens) (Sánchez *et al.,* 2002). Similar findings were obtained by the present study. Minihan *et al*. (2006) found antibiotic resistance profiles of *Campylobacter* isolates to be related to the bacterial origin and not the species. Nevertheless, further work with a big sample size is advisable before a conclusion is made since *Campylobacter* species are reported to correlate with antibiotic resistance profiles to some degree (Olah *et al*., 2006).

Conclusion

Improved management, methods and sanitation are required to reduce the number of birds being colonized by *Campylobacter* significantly and to limit the extent of the cross-contamination among carcasses during processing. The bacterial numbers in flocks and processed carcasses should be monitored regularly. An examination of a bigger sample size is warranted in future to draw a definite conclusion on the antibiotic resistance rate of the bacteria in broiler. It is vital to use antibiotics prudently in broilers to maintain their effectiveness.

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